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A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp. *lycopersici* toxins and fumonisin B₁

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The phytopathogenic fungus *Alternaria alternata* f. sp. *lycopersici* (AAL) produces toxins that are essential for pathogenicity of the fungus on tomato (*Lycopersicon esculentum*). AAL toxins and fumonisins of the unrelated fungus *Fusarium moniliforme* are sphinganine-analog mycotoxins (SAMs), which cause inhibition of sphingolipid biosynthesis *in vitro* and are toxic for some plant species and mammalian cell lines. Sphingolipids can be determinants in the proliferation or death of cells. We investigated the tomato *Alternaria* stem canker (*Asc*) locus, which mediates resistance to SAM-induced apoptosis. Until now, mycotoxin resistance of plants has been associated with detoxification and altered affinity or absence of the toxin targets. Here we show that SAM resistance of tomato is determined by *Asc-1*, a gene homologous to the yeast longevity assurance gene *LAG1* and that susceptibility is associated with a mutant *Asc-1*. Because both sphingolipid synthesis and *LAG1* facilitate endocytosis of glycosylphosphatidylinositol-anchored proteins in yeast, we propose a role for *Asc-1* in a salvage mechanism of sphingolipid-depleted plant cells.

Mycotoxins are a continuous risk in the production and postharvest storage of crops, threatening animal and human health. They can be divided into the nonselective mycotoxins, phytotoxic to a broader range of organisms than the producing fungus infests and in the host-selective mycotoxins, primary determinants of phytopathogenicity (1). One class of host-selective mycotoxins is structurally related to sphinganine (2), a precursor in plant sphingolipid biosynthesis (Fig. 1) (3). Two nonrelated fungi are well known for the production of sphinganine-analog mycotoxins (SAMs): *Fusarium moniliforme*, a fungal pathogen of maize that produces fumonisin B₁ (FB₁) (4) and *Alternaria alternata* f. sp. *lycopersici* (AAL), a tomato pathogen that produces AAL toxins (5). SAMs competitively inhibit *de novo* sphingolipid (ceramide) biosynthesis *in vitro* (see Fig. 1), which leads to a variety of cellular responses, including the accumulation of sphingoid bases in animal cells (6) and plants (7). Besides their role as structural components of biomembranes, sphingolipids can be determinants in the proliferation or death of cells, depending on the type of cells studied (6). SAMs are toxic to all tissues of sensitive tomato cultivars at low concentrations (5, 8) and induce apoptosis in sensitive tomato lines (9). SAMs also induce apoptosis in some mammalian cell lines (2) and are toxic to animals and suspected to be conditionally carcinogenic in humans (4). These data indicate the necessity to understand the mechanism of action of SAMs, which has been performed by studying SAM resistance of tomato.

Until now, mycotoxin resistance of plants has been associated with detoxification and altered affinity or the absence of the toxin targets (1). Resistance or susceptibility to both AAL and SAMs in tomato is determined by the *Alternaria* stem canker (*Asc*) locus (5). It inherits in a single codominant fashion. Plants heterozygous for the *Asc* locus (*Asc*, *asc*) have an intermediate

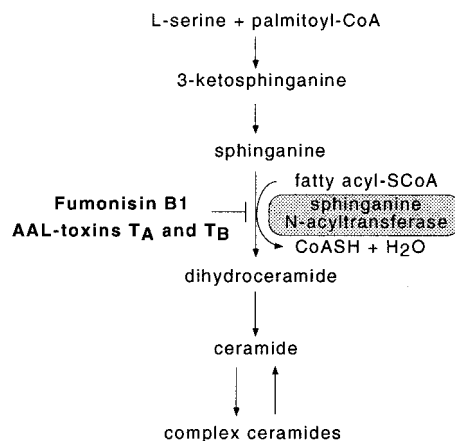


Fig. 1. *De novo* sphingolipid synthesis in plants. This scheme has been adapted from a recent report (3) in which enzyme activities and sphingolipid composition in plants were summarized. The conversion of sphinganine to ceramide is inhibited by FB₁ and AAL toxins. Variations in plant ceramides exist by the presence of hydroxylated or (poly)unsaturated sphinganine as the sphingoid base moiety or by 2-hydroxylated fatty acyl groups. Predominant complex sphingolipids are glycosylceramides and inositolphosphorylceramides. No plant genes involved in this process have been characterized yet.

sensitivity to SAMs. AAL toxins are essential for the pathogenesis of AAL in tomato, because AAL toxin-deficient mutants cannot infest healthy leaves of the *asc* isoline (10). *F. moniliforme*, however, is not pathogenic on the *asc* isoline (8), indicating that FB₁ produced by *F. moniliforme* is not sufficient for virulence on tomato. Our goal was to establish the molecular basis of the resistance and susceptibility of tomato to SAMs. Based on an established genetic analysis of the *Asc* locus (11), we have isolated the *Asc* gene by positional cloning. Sensitivity is

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Abbreviations: AAL, *Alternaria alternata* f. sp. *lycopersici*; SAMs, sphinganine-analog mycotoxins; *Asc*, *Alternaria* stem canker; YAC, yeast artificial chromosome; RT-PCR, reverse transcription-PCR; TM, transmembrane; *LAG1*, longevity assurance gene 1; ER, endoplasmic reticulum; *DGT*, delayed ER-to-Golgi transport gene; GPI, glycosylphosphatidylinositol; EGGAP transport, ER-to-Golgi transport of GPI-anchored proteins; FB₁, fumonisin B₁.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF198177 (*Le Asc-1*), AF198178 (*Le asc-1*), AF198179 (*LAG1 At-1*), and AF198180 (*LAG1 At-2*)].

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associated with a mutation in the *Asc* gene that leads to a predicted aberrant ASC protein, a new plant member of the longevity assurance protein family.

Materials and Methods

Plant Materials. For molecular analysis of the *Asc* and *asc* alleles, Clouse and Gilchrist (12) donated paired tomato lines isogenic and homozygous for the *Asc* locus [the *Asc* (resistant) and *asc* (sensitive) isolines]. For the production of YAC442A, the SAM-resistant tomato line VFNT Cherry has been used by Bonnema *et al.* (13). The SAM-sensitive *asc*, *asc* line *Tester-3* (11) has been used in the functional complementation assay.

Isolation of the *Asc-1* Gene. All nucleic acid manipulations were performed according to Sambrook *et al.* (14) unless otherwise stated. A phage Lambda and a binary plasmid library were produced by partial *Sau3A* digestion of genomic DNA from the YAC442A yeast strain (11), size selection for DNA fragments larger than 10 kb and ligation into *Bam*HI-digested phage Lambda GEM-11 arms (Promega) or pCGN1548 (15), respectively. *Eco*RI DNA fragments (Fig. 2A) from Lambda GEM-11 clones were converted to cleaved amplified polymorphic sequence markers (16) and the approximate positions of the recombinant breakpoints were determined. The clones pBAsc1 and pBAsc2 were isolated from the pCGN1548 library to encompass the *Asc* region within the nearest recombinant breakpoints. Because the pCGN1548 backbone is incompatible with *Agrobacterium rhizogenes*, the pBAsc1 and pBAsc2 clones were further subcloned into the binary vector pGreen029 (details about the pGreen/pSoup binary vector system are available at <http://www.pGreen.ac.uk>) as *Bam*HI and *Asp*718 fragments, resulting in pBAsc101 and pBAsc206, respectively. To get a binary construct from which ORF1 is exclusively expressed, two *Sac*I fragments (encompassing the 3' region of ORF1; 1,766 bp) and an *Apa*I fragment (*Asc-1* upstream region; 4,511 bp) were subsequently deleted from pBAsc206 to obtain pBAsc404 (Fig. 2A). Partial putative ORFs were predicted at <http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html> (17) and further analyzed with the BLAST algorithms at the National Center for Biotechnology Information website (18). Full-length clones from the Newman PRL2 cDNA library were donated by the *Arabidopsis* Biological Resource Center of Ohio State University (Columbus, OH) for 220P10T7 [*Longevity Assurance Gene 1* (*LAG1*) *At-1*] and 92N21T7 (*LAG1* *At-2*) from *Arabidopsis thaliana* ecotype *Columbia*. The tomato DNA inserts of pBAsc1 and pBAsc2 and the above cDNA clones were sequenced in both directions by dye terminator cycle sequencing (Applied Biosystems). The *asc-1* gene was amplified with *Asc*-specific oligonucleotides BASC86 (*Eco*RI tail, forward) 5'-CGGGATC-CCGATCAGTCTTTGTGGTCATCATC-3' and BASC87 (*Bam*HI tail, reverse) 5'-GGAATTCCTGCAATTCATTT-GAACTACAAC-3', subsequently cloned in pGEM-Teasy (Promega), and multiple clones were sequenced in both directions to find differences with the *Asc-1* gene avoiding PCR artifacts. Contiguous DNA sequences were constructed with the SEQUENCHER 3.0 software (Gene Codes, Ann Arbor, MI). Transmembrane (TM) segments were predicted with the TMHMM1.0 server at the Center for Biological Sequence Analysis, Technical University of Denmark (<http://www.cbs.dtu.dk/services/TMHMM-1.0/>).

Hairy Root Functional Complementation Assay. The binary vector pGreen029 or its derivatives carrying tomato DNA inserts were transferred to *A. rhizogenes* LBA9402 together with the helper plasmid pSoup by electroporation and selected on yeast mannitol broth (YMB) (0.4 g/liter yeast extract/10 g/liter mannitol/0.1 g/liter NaCl/0.5 g/liter K₂HPO₄/0.2 g/liter MgSO₄·7H₂O) with 50 mg/liter kanamycin. Hypocotyl explants (3 cm) from

7-day-old *Tester-3* tomato seedlings grown on root medium [half-strength Murashige and Skoog salts with vitamins/30 g/liter sucrose/8 g/liter plant agar (Duchefa, The Netherlands)] *in vitro*, were dipped in an *A. rhizogenes* suspension and cocultivated for 48 h on root medium. Transgenic hairy roots were selected and cleared from *A. rhizogenes* by subculturing on root medium supplemented with 50 mg/liter kanamycin/500 mg/liter carbenicillin/100 mg/liter vancomycin. The presence of the transgenes was verified by PCR and Southern blot analysis. A 95:5% mixture of AAL toxins T_A/T_B was isolated as described (19), and fumonisin B₁ was obtained commercially (Sigma). Hairy root tips were tested for complementation to SAM resistance by determination of root growth (white root cap; 1 cm/day elongation) or death (brown root tip; less than 1 mm/day elongation) on root medium supplemented with 0.07 μ M AAL toxins mixture or 0.7 μ M fumonisin B₁.

DNA and RNA Analysis. Tomato *Asc* isoline plants were grown in pots under standard Dutch greenhouse conditions (20°C, 60% humidity, 15 kilolux light intensity maintained by automated artificial lights) and mature leaves were harvested 3 weeks after emergence of the seedlings. A DNA blot with *Xba*I- or *Hind*III-digested genomic DNA (7.5 μ g) of these leaves was prepared with positively charged nylon membranes (Hybond N⁺, Amersham International) as described (20). The DNA blot was hybridized with a radiolabeled 1.5-kb PCR fragment prepared with the primers BASC86 and BASC87 encompassing the complete *Asc-1* ORF, washed at low stringency (2 \times SSC at 60°C), and analyzed by a PhosphorImager (Molecular Dynamics). Total RNA was isolated from the above leaves in three independent experiments as described (20) and mRNA was subsequently isolated by the Oligotex procedure (Qiagen, Chatsworth, CA). Reverse transcription-PCR (RT-PCR) was performed by reverse transcription of 0.25 μ g of mRNA with Expand by using the instructions of the manufacturer (Boehringer Mannheim). Sequences of ORF1 and ORF2 were determined by amplification of the cDNA with the primers BASC86 and BASC87 for ORF1, and with BASC76 (forward) 5'-AGAGGAGCTGACATTTG-3' and BASC77 (reverse) 5'-CCACAAATCTTGTCCTATCT-3' for ORF2. In competitive RT-PCR amplifications of *Asc* (21), equal volumes of the cDNA preparation, a logarithmic dilution series of pBF103 [a pGEM3z (Promega)] plasmid containing the 5.0-kb *Eco*RI fragment with a complete genomic copy of *Asc-1* and the *Asc*-specific oligonucleotides BASC88 5'-GCTGCGCAGATAATTCATCA-3' (forward) and BASC89 5'-TCCGTTCTCTGTATATCC-3' (reverse) were used.

Leaflet Bioassay. Detached leaflets of greenhouse-grown *Asc* and *asc* isoline plants were incubated in triplicate in 9-cm Petri dishes on filter papers (Schleicher & Schüll #595, Dassel, Germany) saturated with 0.1% (vol/vol) Tween-80 (control), supplemented with 0.2 μ M AAL toxins mixture, 100 μ g/ml (wt/vol) ceramide type III or both. Ceramide type III (Sigma C2137) was dissolved at 50°C in 96% ethanol containing 0.1% Tween-80 before making dilutions. Tween-80 did not induce necrotic symptoms and was essential to keep the lipophilic ceramides in solution. Symptoms were observed 72 h after incubation in a climate cabin (22°C) in the light (3 kilolux).

Results

Functional Complementation of the *asc* Mutant. Position-based molecular isolation of *Asc-1* (Fig. 2A) was initiated from adjacent chromosome markers TG134 and TG442 and a plant population with recombinations around the *Asc* locus (11). This approach enabled the isolation of a YAC442A encompassing the *Asc* locus. In addition, the YAC442A molecular markers P6 and C18 have been produced, residing at one and three recombinations from

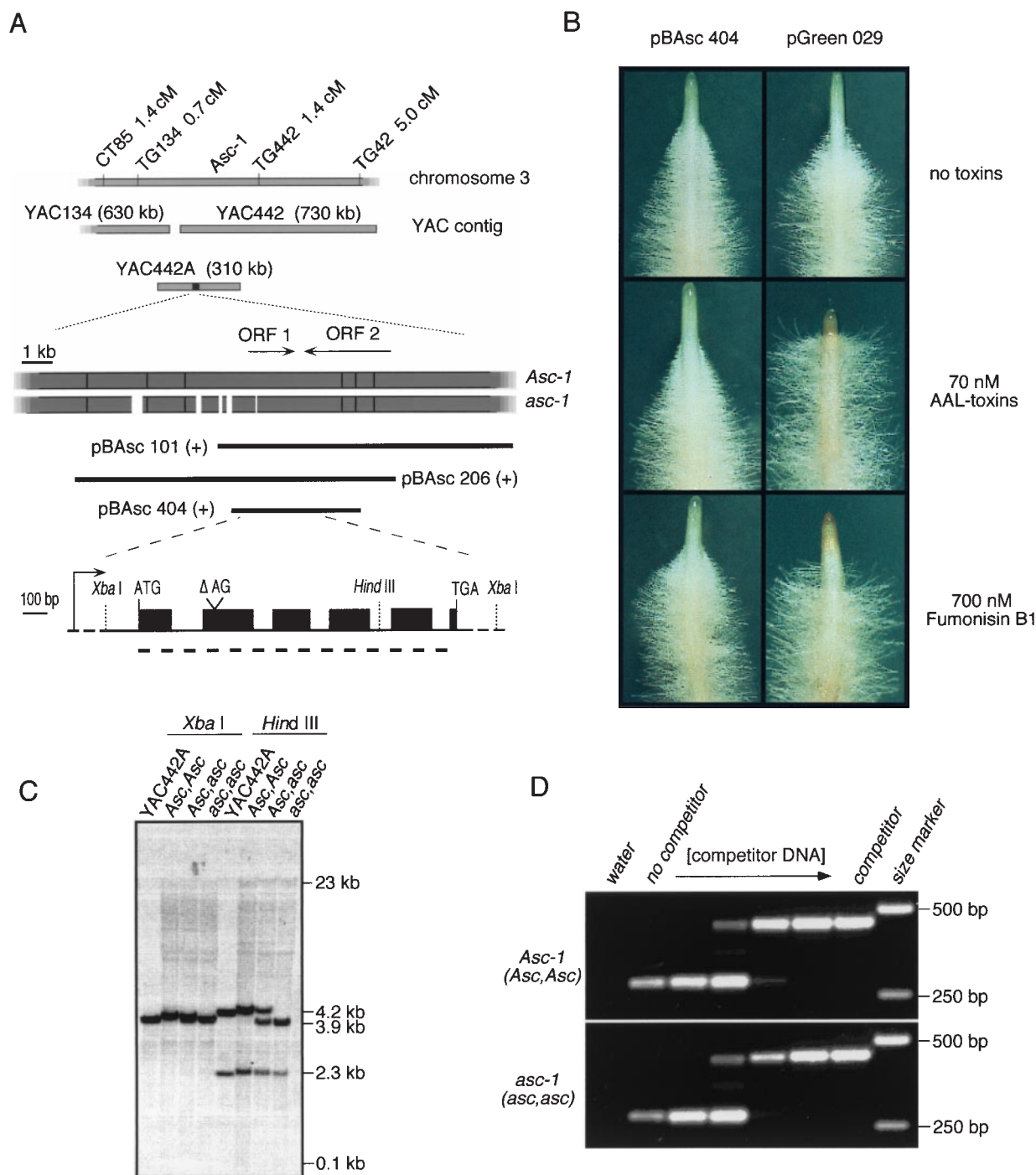


Fig. 2. Molecular isolation and characterization of *Asc-1*. (A) Isolation of *Asc-1*. Genetic distance of chromosome markers are in centimorgans (cM); overlapping YACs are light gray boxes, in the detail of YAC442A (dark gray) are the *EcoRI* fragments positioned at zero recombinants. The corresponding chromosome segment in the *asc*, *asc* isoline (*asc-1*) contains deletions (blank gaps). Tomato DNA fragments used in the *A. rhizogenes*-mediated transformation are black lines. Only DNA constructs which give functional complementation in the hairy root bioassay (+) are depicted. ORF1 is *Asc-1* (2.5 kb), which contains six exons (black boxes). The second exon of the *asc-1* gene contains a 2-bp deletion (Δ AG). (B) Hairy root bioassay with two representative transformants of pGreen029 (empty binary vector) and pBAsc404 (containing *Asc-1*) on SAMs. (C) Genomic DNA blot of the *Asc* isolines and YAC442A hybridized with the coding region of the *Asc-1* gene (dashed line in A). The deletions in the *asc-1* promoter region are detected by a shift of a 4.2- (*Asc*) to a 3.9-kb (*asc*) *HindIII* fragment. (D) Competitive RT-PCR for visualizing relative mRNA levels of *Asc-1* and *asc-1* in leaves of the *Asc* isolines. Experiments were repeated three times from individual plants with similar results. The upper fragment represents the 457-bp fragment amplified from added competitor genomic *Asc-1* DNA; the lower band represents the *Asc-1*-specific 289-bp cDNA fragment.

Asc, respectively (11). Chromosome walking from P6 and C18 in a Lambda phage YAC442A library produced a 70-kb contiguous stretch of Lambda clones spanning the *Asc* locus. By positioning the nearest recombinant breakpoints adjacent to *Asc*, the

genomic region to be used for functional complementation was confined to 17 kb (Fig. 2A). Functional complementation by YAC442A DNA fragments in a sensitive *asc*, *asc* background was accomplished by the induction of *A. rhizogenes*-induced trans-

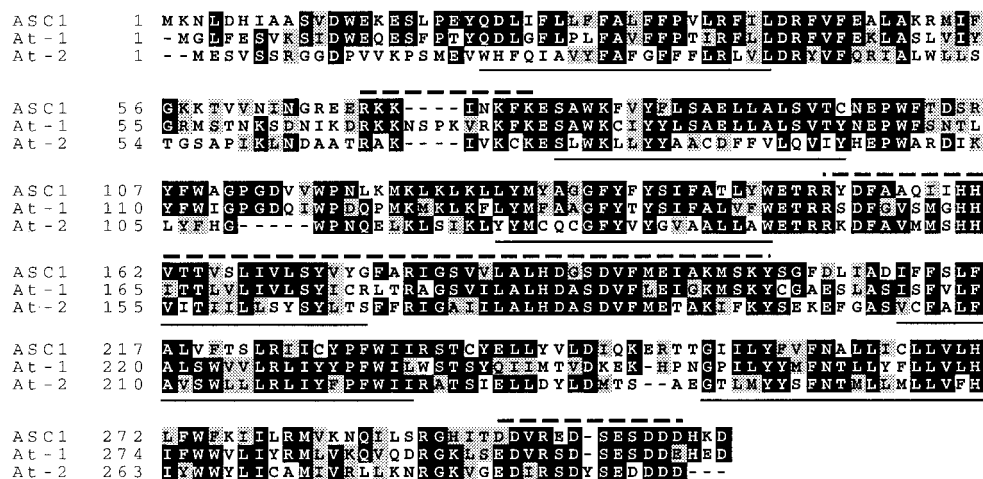


Fig. 3. Amino acid alignment of the *L. esculentum* ASC1 (Le) and *A. thaliana* LAG1 (At-1 and At-2) protein homologs. The ASC-1 and LAG1 At-1 homologs are 53% identical (black) and 73% similar (gray). The predicted TM spanning helices are underlined, the basic domain at amino acids 57–83 (in ASC1), the LAG1 motif at amino acid 151 (in ASC1), and the C-terminal acidic domain are indicated with dashed lines.

genic hairy roots and subsequently testing root viability on SAMs. Before transgenic roots were tested, it was crucial to optimize the SAM concentrations to discriminate between hairy roots induced with a wild-type *A. rhizogenes* on explants of the *Asc*, *asc* and the *asc*, *asc* tomato genotype. In general, SAM concentrations above 1 μ M lead to growth inhibition of *Asc*, *asc* hairy roots, whereas SAM concentrations below 20 nM had no effects on *asc*, *asc* hairy roots. For AAL toxins, a concentration of 70 nM and for FB₁, a concentration of 700 nM was found to be optimal for discrimination. This is in agreement with the observation that FB₁ is approximately 20 times less phytotoxic for detached tomato leaflets than AAL toxins (8). Two genomic DNA fragments (pBAsc102 and pBAsc206) were identified that, on transformation, resulted in the generation of SAM-resistant roots at the discriminating concentrations, indicating that SAM resistance is a dominant property (Fig. 2A). Roots transformed with the control vector pGreen029 could be distinguished from SAM-resistant roots by the inhibition of root elongation, necrosis of the root tip, and deformation of root hairs (Fig. 2B).

Identification and Characterization of the *Asc* Gene. By sequencing of the pBAsc102 and pBAsc206 plasmid inserts which complemented the *asc*, *asc* mutant phenotype, two closely linked putative ORFs potentially encoding *Asc* have been detected in the 17-kb contiguous DNA sequence. Both ORFs are expressed in resistant tomato lines, because the corresponding mRNAs have been detected by RT-PCR analysis. DNA sequence determination of RT-PCR products of ORF2 did not reveal any differences between the *Asc* isolines (data not shown). To obtain a binary construct from which ORF1 is exclusively expressed, a deletion construct was made from pBAsc206 to obtain pBAsc404 (see Fig. 2A). Complementation assays of pBAsc404 confirmed that ORF1 driven by its endogenous promoter (*Asc-1*; Fig. 2B) mediates SAM resistance, demonstrating that ORF1 encodes the ASC1 protein. Analysis of DNA blots indicated that *Asc-1* is a single-copy gene and one homolog is present in the *asc* isolate, further referred to as the *asc-1* gene (Fig. 2C). Because the *asc* isolate has no obvious visible phenotype in the absence of SAM, this gene does not appear to be essential for plants under standard greenhouse conditions. Intron positions in *Asc-1* were determined by comparison of the cDNA with the genomic DNA sequences, because several positions were not predicted by GENEFINDER algorithms. The predicted ASC1 36-kDa protein

contains six TM helices, a basic domain between TM1 and TM2, and a C-terminal acidic domain (Fig. 3). Expression of *Asc-1* and *asc-1* could not be detected on blots containing mRNA isolated from leaves, flowers, and immature fruits (data not shown). However, in a competitive RT-PCR amplification with *Asc*-specific oligonucleotides, the expression of *Asc-1* and *asc-1* was found to be equal (Fig. 2D). To further investigate the difference between SAM-resistant and -sensitive genotypes, PCR with *Asc-1*-specific oligonucleotides and subsequent sequencing revealed five deletions ranging from 4 to 133 bp (in total 300 bp) in the promoter region of the *asc-1* gene. These deletions are visible as a single restriction fragment length polymorphism on Southern blots of *Hind*III-digested genomic DNA of the *Asc* isolines (Fig. 2C). Apparently, these deletions do not affect transcription of *asc-1*. The most significant mutation detected in the sensitive genotype is a 2-bp deletion in the second exon of the *asc-1* gene that leads to a premature stop codon in exon 2 (Δ AG in Fig. 2A). Thus, sensitivity of *asc*, *asc* plants to SAM is likely associated with a truncated aberrant 10-kDa protein, which provides additional support that ORF1 mediates SAM insensitivity. The intermediate SAM sensitivity of heterozygous *Asc*, *asc* plants can be explained by a gene-dose effect, assuming that the steady-state level of *Asc-1* mRNA determines the abundance of ASC1 protein.

***Asc* Belongs to the Longevity Assurance Gene Family.** *Asc-1* has no homology to any published plant disease resistance gene, which has been expected because resistance is based on insensitivity to SAMs, as opposed to the specific response to pathogen-encoded elicitor molecules in gene-for-gene host-pathogen interactions. Interestingly, *Asc-1* is homologous to the *Saccharomyces cerevisiae* LAG1 that has been associated with life span in yeast (22). By accessing public DNA databases for expressed sequence tags and full-length cDNAs, LAG1 homologs were found to be transcribed in fish, mammals, insects, higher fungi, and monocotyledonous and dicotyledonous plants (Fig. 4). The cDNAs for two *A. thaliana* homologs (LAG1 At-1 and At-2) were donated by the Arabidopsis Biological Resource Center and sequenced. Although the overall homology between the known LAG1 proteins among distantly related species is limited (for instance, 26% identity and 46% similarity for the *S. cerevisiae* LAG1 with ASC1 over 141 amino acids), they share a similar six or seven TM helices profile and the LAG1 motif (23). In addition, a C-

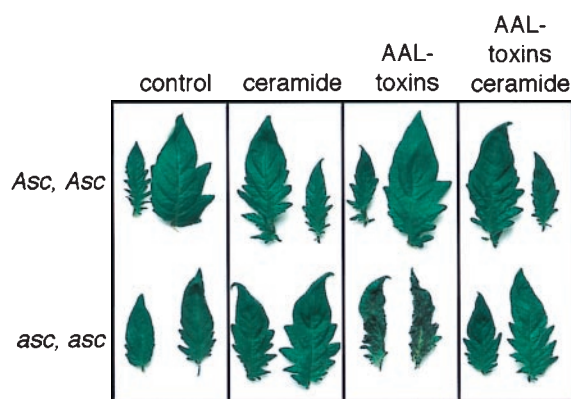


Fig. 6. Ceramide type III rescues AAL toxin-induced apoptosis. The control incubation or ceramide do not result in cell death in detached leaflets of both *Asc* isolines. At the concentrations used, AAL toxins only induce interveinal cell death and loss of turgor in the SAM-sensitive *asc, asc* isolate. The cell death is counteracted by the addition of exogenous ceramides during toxin treatment.

lipids which enables yeast to grow without making sphingolipids (28). In analogy, *Asc-1* may act as a salvage mechanism to restore EGGAP transport by encoding a similar enzyme, although SLC1 has no significant homology with ASC1. Sphingolipid precursors accumulate in both AAL toxin-treated *Asc* and *asc* isolate plants (7), but it has not been tested if complex ceramides are depleted. It is thus not clear which of the two events is tolerated in SAM-resistant tomato plants. Interestingly, ceramides rescue detached sensitive tomato leaves from SAM-induced apoptosis (Fig. 6), suggesting that only ceramide depletion is responsible for SAM toxicity in the *asc* isolate. Consequently, ceramides may not be depleted completely in the *Asc* isolate as a result of an enzymatic activity encoded by *Asc-1*. The presence of both SAM-insensitive and -sensitive sphinganine-*N*-acyltransferase

activities has been reported for beans and squash (3). Alternative to the first hypothesis, ASC1 could thus be a SAM-insensitive sphinganine-*N*-acyltransferase, assuming the presence of a SAM-sensitive sphinganine-*N*-acyltransferase encoded by an *Asc-1* homolog in both *Asc* isolines. Because ceramide precursors such as sphinganine (Fig. 1) accumulate in the *Asc* isolate after SAM exposure (7), the relative contribution of a possible SAM-insensitive sphinganine-*N*-acyltransferase to the overall *de novo* ceramide synthesis should be limited. The possible presence of an *Asc-1* homolog is in contrast with the apparent single-copy nature of *Asc-1* (Fig. 2D). However, in *S. cerevisiae*, *C. elegans*, and *A. thaliana*, at least two copies of LAG1 homologs exist (Fig. 3). The human and *C. elegans* LAG1 genes could not be isolated by using the yeast LAG1 gene as a probe, likely because of limited nucleic acid homology (23). By using lower-stringency hybridization conditions, however, a single *Asc-1* homolog has been discovered in both *Asc* isolines (not shown). In contrast to *Asc-1* (Fig. 1C), no restriction fragment polymorphisms were detected for this homolog, which may be redundant with the function of *Asc-1*, analogous to the *DGT1* gene in yeast (23, 25). We propose that ASC1 compensates for a depletion in ceramides after SAM exposure and thereby protects the cell from arrest of EGGAP transport. In conclusion, the *LAG1*-like *Asc-1* gene rescues tomato hairy roots from SAM-induced cell death which opens the way to study the role of sphingolipid-dependent transport of GPI-anchored proteins in the apoptosis of plant cells.

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